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PRINCIPAL INVESTIGATOR: Stephen M. Lasley, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois at Chicago
Chicago, IL 60612-7227

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Lasley, Stephen, Ph.D.

Email: sml@uic.edu

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14. ABSTRACT
The chemical properties and high density of depleted uranium (DU) render the metal well suited for military purposes, but knowledge of DU neurotoxicity and its treatment is lacking. This project is designed to test the hypothesis that *long-term administration of an anti-oxidant agent and/or an NMDA receptor antagonist will reduce neurotoxicity resulting from chronic exposure to DU.* This hypothesis is based on previous observations in rats chronically exposed to DU, and reflects the anticipation that specific pharmacological agents will reverse signs of DU-induced oxidative stress. As prescribed by the Statement of Work, efforts continued in year 2 on Tasks 1 (drug therapies to reverse DU-induced neurotoxicity) and 2 (brain DU concentrations) utilizing experimental groups (0, 300, and 600 mg DU) exposed for 9 months. Task 1 is nearing completion, but at this point the NMDA receptor antagonist has not demonstrated neuroprotective effectiveness. Progress has been achieved on Tasks 2-4, and remaining subject cohorts will be analyzed in year 3. Thus, progress is proceeding according to the schedule specified in the Statement of Work.

15. SUBJECT TERMS
depleted uranium, glutamate release, military disease, hippocampus, oxidative stress, neuroprotectant drugs

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INTRODUCTION

The chemical properties and high density of depleted uranium (DU) render the metal well suited for military purposes. The U. S. Army utilizes DU for tank armor and in munitions, deployed such weapons in Gulf War I, and is currently deploying them in Iraq. However, knowledge of DU neurotoxicity and its treatment is lacking despite reports of exposed cohorts exhibiting neurocognitive dysfunction. Research in chronically exposed rats has reported alterations in hippocampal synaptic transmission, suggesting DU-induced decreases in neuronal excitability (1). This project examines potential treatment options to address neurotoxicity from chronic DU exposure. On the basis of previous observations the bases of DU neurotoxicity are proposed to be cellular oxidative stress and the consequent increased production of reactive oxygen species, leading to decreased glutamate uptake and increased synaptic glutamate concentrations in conjunction with NMDA receptor up-regulation. Uranium-induced oxidative stress has previously been reported in rat kidney, testis, and lung (2-3). Studies will identify various biochemical markers of metal-induced oxidative stress in hippocampal and cortical tissue, and in combination with enhanced extracellular glutamate and NMDA receptor activity will provide three components of DU neurotoxicity for assessment of therapeutic efficacy. *It is hypothesized that long-term administration of an anti-oxidant agent and/or an NMDA receptor antagonist will reduce DU neurotoxicity.* These studies will provide critical information on which to base new treatments for exposed Gulf War veterans.

BODY

As prescribed in the approved Statement of Work, project activities in year 2 primarily addressed Tasks 1 and 2, but also encompassed initiation of exposure in most of the animals to be utilized in Task 3. A description of these efforts and the resulting progress toward each objective is provided below.

Task 1 concerns demonstration of the efficacy of chronically administered drug therapies to reverse DU-induced elevations in extracellular glutamate in superfused hippocampal slices from chronically exposed animals. The project includes a control group and low (300 mg load) and high dose (600 mg load) DU exposure conditions, but utilizes a vehicle and three drug-treated groups (memantine or riluzole or a combination) for each exposure level. This design results in a 3 exposure level × 4 drug condition matrix with 8 animals/cell (96 animals/cohort), thus maximizing the ability to discriminate the actions of the therapeutic agents on the proposed measures. Drugs are administered via osmotic minipumps (Alzet) surgically inserted subcutaneously. Adult male Sprague-Dawley rats are implanted intramuscularly with DU pellets at 70-80 days of age; beginning at 3-4 months of age they are placed on food restriction so that their maximal body weight does not exceed 475-525 grams. After 7 months exposure 28-day minipumps are implanted and replaced once to cover the period up to 9 months when exposure is terminated and testing conducted. This is an appropriate interval for drug administration as the slope of the increase in DU concentration is greater during this period than prior to 6 months exposure. The minipumps are filled with drug solutions of 30 mg/ml memantine (3.6 mg/kg/day) and/or 10 mg/ml riluzole (1.2 mg/kg/day). Besides its potential usefulness as an uncompetitive NMDA receptor antagonist, memantine also has been reported to have neuroprotectant value via induction of brain-derived neurotrophic factor and its receptor (4-6),

making the drug of particular interest for this project. Blood samples are collected from sufficient animals prior to sacrifice to establish the plasma drug levels achieved and validate the drug administration protocols. Table 1 summarizes progress on this Task to date (~80% complete) by listing within the experimental design the numbers of animals that have completed exposure and drug treatments. Ultimately, each cell of the matrix will contain 7-9 animals. Table 2 lists the plasma drug levels achieved over a period of 7-8 weeks with this dosing regimen.

Table 1

Number of Animals Completing Exposure and Testing

DU Exposure, mg pellets	Vehicle	Memantine	Riluzole	Memantine & Riluzole
0	9	7	6	6
300	6	7	7	6
600	6	6	7	4

Table 2

Plasma Drug Levels, ng/ml		
Group	**MEM**	**RIL**
Vehicle	**- 0**	**-- 0**
Memantine	**28.1 ± 3.4**	**--**
Riluzole	**--**	**41.1 ± 3.0**
Memantine & Riluzole	**17.2 ± 1.2**	**24.5 ± 2.3**

Values are mean ± SEM, N = 8-9. Blood sampled from jugular vein 7-8 weeks after drugs instituted. MEM = memantine; RIL = riluzole.

Evidence from previous work has indicated that the effects of chronic exposure to DU on depolarization-induced hippocampal extracellular glutamate are a combination of acute UO_2^{+2}-dependent inhibition of release and an opposing slowly developing increase in extracellular transmitter, perhaps due to metal-induced mitochondrial and glutamate transporter dysfunction. An additional component of the observed neurotoxic response is a substantial up-regulation of NMDA receptor density. Data from the current project collected on Task 1 to date are shown in Figures 1-3. The results shown display depolarization-induced glutamate release from K^+-stimulated hippocampal slices after exposure to DU for 9 months in conjunction with continual administration of memantine (Figure 1), riluzole (Figure 2), or the combination (Figure 3) for months 8-9 via osmotic minipumps.

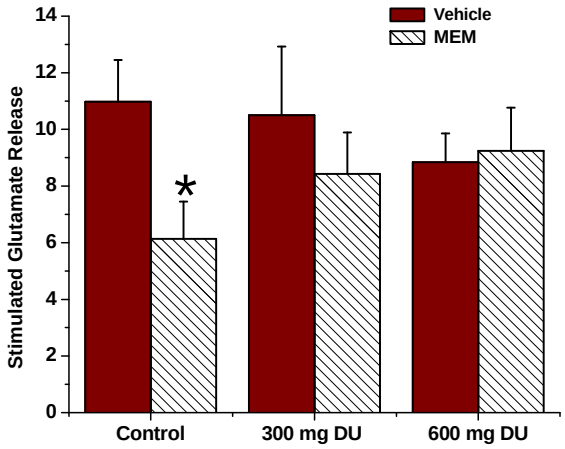

Figure 1. Evoked glutamate release from K^+-stimulated hippocampal slices after exposure to DU for 9 months and continual administration of memantine for months 8-9 via osmotic minipumps. Values are expressed as mean ± SEM (N = 6-9/group) of the area under the curve normalized to 1.0 and summed across the peak response intervals. * p <0.05 compared to the paired group receiving vehicle only in the minipumps.

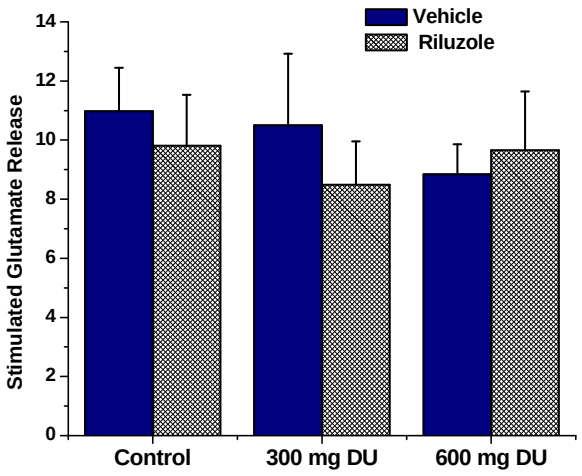

Figure 2. Evoked glutamate release from K^+-stimulated hippocampal slices after exposure to DU for 9 months and continual administration of riluzole for months 8-9 via osmotic minipumps. Values are expressed as mean ± SEM (N = 6-9/group) of the area under the curve normalized to 1.0 and summed across the peak response intervals.

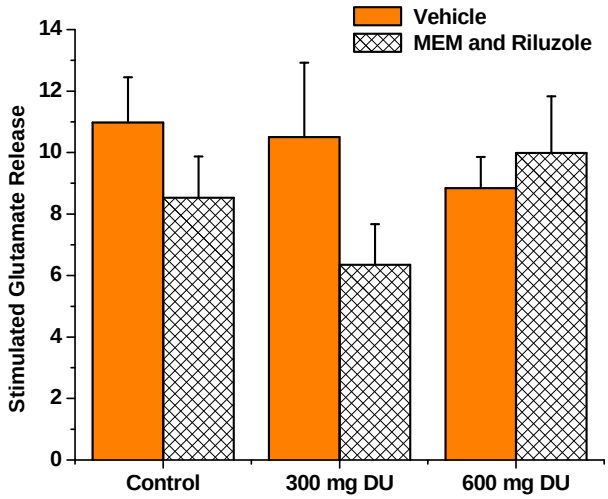

Figure 3. Evoked glutamate release from K^+-stimulated hippocampal slices after exposure to DU for 9 months and continual administration of memantine and riluzole for months 8-9 via osmotic minipumps. Values are expressed as mean ± SEM (N = 6-9/group) of the area under the curve normalized to 1.0 and summed across the peak response intervals.

While this dataset is not fully complete, two preliminary observations are noteworthy. First, the enhancement of evoked extracellular glutamate concentrations found after 15-17 months of DU exposure in earlier work was not discriminated after 9 months of exposure, suggesting that longer periods of administration are necessary for the glutamate transporter dysfunction to become the dominant effect over the opposing UO_2^{+2}-dependent inhibition of release. Second, memantine significantly diminished evoked extracellular glutamate in control vehicle-treated animals, but had no effect on groups chronically exposed to DU. Administration of riluzole and the memantine/riluzole combination did not alter glutamate responses to high K^+ in any groups.

The bases for the attenuation of stimulated glutamate release by memantine in control animals, and the absence of this effect in the DU groups requires further investigation. Since memantine is an antagonist at NMDA receptors, the DU-induced receptor up-regulation may result in a smaller proportion of inhibited NMDA receptors in the exposed than in the control groups, and thereby cause reduced drug effectiveness in DU animals. However, memantine also exhibits other pharmacological actions that must be considered, such as inhibiting nicotinic receptors (7) and enhancing neurotrophic factor function (e.g., 6).

It is possible that the doses of memantine/riluzole utilized were not adequate to produce more consistent effects in the control and DU groups. If this were true, another route of administration likely would have to be chosen, since the concentrations of drug applied through the osmotic minipumps approached their solubility limits. A longer duration of drug administration may also have been more effective in countering DU neurotoxicity, but such a regimen would have less relevance to the Gulf War cohort of exposed soldiers who remain essentially untreated at this time.

The remaining animals to be tested to complete Task 1 are primarily replacements for surgical losses or animals discarded from the study for other reasons. In addition, independent control and DU groups are being generated that will not receive minipumps containing vehicle, and thus will more closely simulate the exposure of these groups in the earlier project.

Task 2 consists of determination of DU concentrations in brain tissue of chronically exposed animals at durations corresponding to the beginning (7 months) and end (9 months) of the drug therapies. Characterization of the protocol in this Task is designed to provide context to the experimental findings generated under the other Tasks. The determination of hippocampal uranium levels will be performed by inductively coupled plasma-mass spectrometry (ICP-MS) analysis by a commercial laboratory (Elemental Analysis, Inc., Lexington, KY). This methodology has proven more sensitive and reliable for this sample matrix than alternative approaches, and this vendor has previously provided reliable determinations. The DU used in this project consists of 30 mg pellets (1 mm diameter × 2 mm length) obtained from Aerojet Ordnance Tennessee (Jonesborough, TN), and are sterilized prior to use. Ten pellets are implanted in the gastrocnemius muscle of each thigh of 70-80 day old male rats. The design includes three exposure groups: a high dose group in which all pellets are DU (600 mg load), a low dose group receiving 10 pellets of DU (300 mg load), and a control group which received 20 tantalum pellets (0 mg load). The low dose group also receives 10 pellets of tantalum. Tantalum is an essentially inert heavy metal widely used in medical prostheses. The group size (N = 6) is sufficient to characterize the exposure protocol and provide general measures of metal uptake. At this point all tissue samples for these analyses have been harvested and are awaiting shipment to the analytical laboratory.

In order to maximize the efficiency of use of the last cohort of animals Tasks 3 and 4 will both be performed on brain tissue harvested from these rats. Task 3 concerns assessments of biochemical markers of DU-induced oxidative stress in forebrain cortical tissue and the ability of drug therapies to reverse the changes in these measures, and work has begun to set up these assays. ^{3}H-Glutamate uptake into midbrain cortical slices is being measured as a focused assessment of the integrity of the neurotransmitter transport process. The determination of

biochemical markers will utilize commercially available kits, and analyses of other markers – e.g, F_2-isoprostanes – may be performed in conjunction with those originally proposed – catalase and glutathione peroxidase activities. Task 4 will quantify DU-induced elevations in NMDA receptor binding density and the ability of drug therapies to reverse these measures in hippocampal membrane preparations using radioligand binding. DU exposure in this cohort of animals is nearing an end, and testing will begin shortly.

Cost overruns have posed continuing problems throughout the project. These charges are primarily traceable to the higher costs of DU pellets than in previous work, the high expense of the osmotic minipumps which cannot be refilled over the period of drug administration but must be replaced, and the unbudgeted charges for having plasma drug levels determined by an analytical laboratory. The need for the latter analyses to establish the plasma drug levels achieved and to validate the drug administration protocols was not realized until after the GWIRP award had been made and work begun. The tissue analyses for DU to complete Task 2 (cost of ~$10K) have been delayed until funds could be identified to cover these expenses. Nonetheless, it is anticipated that all aspects of the Statement of Work will be completed by the end of the third project year.

KEY RESEARCH ACCOMPLISHMENTS

Considerable effort has been invested to optimize the surgical procedures for DU pellet and osmotic minipump implants, particularly since the latter had to be replaced once in each animal at the 8 month exposure interval. The consistency and reliability of the drug administration regimen is critically important in being able to demonstrate a neuroprotective/antioxidant effect for memantine and/or riluzole, and plasma drug determinations support the other neurochemical tests being conducted.

Performance of the complex experimental design involved in Task 1 is nearing completion, but because of the long DU exposure duration, there are no definitive and finalized results to be reported at this time. Nonetheless, the project is progressing according to schedule.

REPORTABLE OUTCOMES

No Tasks have been fully completed at this time. However, preliminary reports of the work performed on Task 1 have been reported in abstract form at the 2009 Military Health Research Forum (August 31 - September 3) and Society for Neuroscience (October 17-21) meetings.

CONCLUSIONS

Summaries of the progress and its importance as a scientific product are included in the preceding sections **Body** and also in **Key Research Accomplishments**. No conclusions on the effectiveness of memantine and/or riluzole to reverse the effects of DU-induced neurotoxicity can be stated with finality at this time. The chronic DU exposure and drug administration protocols are being established by analytical determinations to be conducted during years 2 and 3 of the project.

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APPENDICES

None

SUPPORTING DATA

None